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# Virus Reviews and Research

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### Research Article

## INFECTION IN SOYBEANS AND ON MULTIPLE HOST PLANTS IN PUERTO RICO BY AN ISOLATE OF COWPEA MILD MOTTLE VIRUS.

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### ABSTRACT

Seed companies in the USA grow winter soybean (*Glycine max* L. Merr.) nurseries in Puerto Rico to advance their breeding programs and seed increase. However, the soybean nurseries are being threatened by a viral disease that cause stunting, leaf and stem necrosis and shoot wilting that leads to death of the whole plant. In the present study transmission assays were conducted using stem grafting, mechanical inoculation, and whiteflies (WFs), *Bemisia tabaci* (Gennadius). Cultivated and wild host plants infested by WFs were surveyed at Dow AgroSciences Research Station and nearby farms at Santa Isabel, Puerto Rico. Based on previous report in Brazil of similar disease caused by an isolate of Cowpea mild mottle virus (CpMMV), a *Carlavirus*, these samples were screened for the presence of the *Carlavirus* using enzyme-linked immunosorbent assays (ELISA) and RT-PCR using *Carlavirus*-specific primers. The results showed that all the transmission assays expressed the viral symptoms on soybean plants on which the tests were done. Out of the 19 plant species surveyed, 8 species gave positive results for the ELISA test. The RT-PCR also successfully amplified a 300 bp fragment from these ELISA positive samples. Additionally, transmission electron microscopy revealed feather-like aggregates of presumed virions in the cytoplasm, characteristic for many members of the genus *Carlavirus*. This virus infecting soybean and other plants in Puerto Rico is considered an isolate of the CpMMV. This study underlines the importance of controlling WFs and weed species that serve as reservoirs both for the vectors and the virus.

**Keywords:** *Carlavirus*, Soybeans, RT-PCR, ELISA, virus transmission.

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### INTRODUCTION

Soybean (*Glycine max* L. Merrill) is one of the most important crops grown throughout the world for human consumption, animal feed, oil production, confectionaries, and other purposes. Weeds, insect pests, and diseases are the major biotic stresses that limit soybean production. In recent years attempt has been made to develop stress tolerant/resistant and high yielding soybean varieties by different biotechnology companies. Soybean is cultivated in Puerto Rico in winter nurseries to advance breeding programs by several seed companies. However, this effort is being constrained by a virus-like disease that cause dwarfing, shoot die back, and stem necrosis of soybean nurseries that makes the damage

intolerable. Symptoms of leaf vein and stem necrosis and plant stunting were observed in soybean experimental plots in the municipalities of Juana Diaz, Santa Isabel, Isabella and Guayanilla, in Puerto Rico. Observations in a winter nursery in Santa Isabel showed 100 percent incidence in some soybean lines, indicating the potential of damage of this disease in soybeans. Symptoms were first observed by the senior author in May 2005 and were associated with flower dropping and poor development of the pods. Seeds harvested from infected plants showed severe deformation and reduced weight compared to the seeds from healthy plants (Rodrigues, unpublished data).

Whiteflies (WFs) (Homoptera: Aleyrodidae) are one of the most common potential vectors found associated to soybean in Puerto Rico (Perez-Sackett et al., 2011). Viral disease associated to similar symptoms and transmitted by *Bemisia tabaci* (Gennadius), on soybeans

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has been reported from Brazil. Studies conducted to identify the causal agent indicated that the virus is an isolate of *Cowpea mild mottle virus*, a whitefly-vectored *Carlavirus* (Almeida et al., 2005). Similar studies in Iran identified a virus that caused mild mosaic symptoms on soybean as an isolate of the CpMMV (Tavasoli et al., 2009). Soybean is susceptible to infection by several viruses, which substantially reduce yield and quality and it is known that soybean is a natural host for 35 potentially important viruses (Edwardson et al., 1991). CpMMV is one of the 60 different viruses known to be transmitted by *B. tabaci* (Markam et al., 1994), which can cause severe economic losses.

Studies conducted on vectors and hosts of *Carlavirus* indicated that the virus is not transmitted by aphids that have a similar feeding habit with that of WFs (Almeida et al., 2005). Moreover, the virus may be infecting, but is not associated to any visible symptoms in some host plants like *Vigna radiata* (L.) R. Wilcz, *V. aconitifolia* (Jacq.) Marechal, and tomato (*Lycopersicon esculentum* L.) (Tavasoli et al., 2009).

According to Iwaki et al. (1982) the transmission of putative *Carlavirus* by WFs is closer to semipersistent than nonpersistent manner. The source of the virus for spreading into crops could be found among plants in the crop (internal sources) or outside borders (external sources) (Harris, 1983). Usually wild plants, volunteers, and perennials may serve as overwintering or over summering hosts of the virus (Bos, 1981). In the southern Puerto Rico, where soybean winter nurseries are located, several species of alternate hosts of WFs are available year round which could serve as permanent source of inoculum for the virus into soybean fields. The detection of pathogenic microorganisms within alternate hosts is important in conducting epidemiological studies and developing control strategies (Hoy, 2003). The objectives of the present study were: 1) To characterize the virus-like agent associated to soybean by conducting transmission assays, electron microscopy, ELISA and RT-PCR using *Carlavirus* specific primers; 2) To survey host plants that serve as host for the vectors (WFs) and the virus itself into soybean fields.

## MATERIAL AND METHODS

### Virus and whitefly samples.

The whitefly and virus samples were collected from Dow AgroSciences (DAS) Research Station and near by farms in Santa Isabel, Puerto Rico from soybean plants. Whitefly and virus cultures were established on soybean plants in screen houses.

### Mechanical inoculation, stem grafting, and vector transmission assays.

Mechanical inoculation, stem grafting, and WF mediated transmissions were conducted using the soybean line 2053A grown in insect-proof cages.

For mechanical inoculation, soybean leaves from virus infected plants were homogenized in sodium phosphate buffer 0.1 M (pH=7.0) (BioBuffer Solutions, Inc. U.S.A.) using mortar and pestle. Then the homogenate was rubbed using carborundum on the upperside of leaves of healthy soybean plants grown on pots. For the stem grafting experiment, healthy soybean shoots (scions) were grafted onto virus infected soybean root stocks. In the case of WF mediated transmission, healthy plants were grown in a cage and 20 adult WFs that have been feeding on symptomatic soybean plants were introduced into each cage. In all cases, viral symptoms were recorded starting two to three weeks after transmission attempts. Two hundred seedlings originated from seeds collected from pods of infected plants were evaluated in greenhouse regarding seed transmission of the virus.

### Transmission Electron Microscopy.

Symptomatic soybean leaf tissues from field and experimentally transmitted were pre-fixed in Karnovsky's fixative and shipped to the microscopy laboratory at Sao Paulo University, Piracicaba campus, state of São Paulo, Brazil, where they were processed following standard protocols for transmission electron microscopy (Milne and Lesemann 1984)

### Host plant survey and virus detection assays.

A total of 18 commonly grown cultivated and wild plant species were surveyed in DAS and nearby farms (Table 1). Leaves of host plants that were found infested with WFs were collected in plastic zip lock bags that were placed in blue ice and transported to University of Puerto Rico, Agricultural Experimental Station, Rio Piedras, where they were processed and analyzed. Plant specimens were taken for species identification (Torres and Laracuenta, 2002).

### ELISA:

Samples were processed using commercial ELISA kit with polyclonal antibody for *Cowpea mild mottle virus* (CpMMV) (DSMZ Company, Germany). Procedures were carried out according to the manufacturer's recommendation and as described in Clark and Adams (1977). For the negative control, healthy soybean leaves extracted in general extraction buffer were used. ELISA results were reported as positive if the absorbance was at least twice the value of the negative control.

### RNA extraction and reverse transcription polymerase chain reaction (RT-PCR):

RNA from leaf samples was extracted using RNeasy Plant Mini Kit (Qiagen Inc., CA, U.S.A.). Leaf tissue (100 mg leaf/plant) was homogenized using a refrigerated mortar and pestle on ice. A total of 4-8 plants per species were individually sampled and the leaves were homogenized in phosphate buffer and then 500 µl the liquid extract was transferred into sterile microcentrifuge

tubes. The extraction process was completed as described in the extraction kit. The RNA was stored at -20°C until RT-PCR was performed.

A portion of the coat protein subunit gene was amplified in a RT-PCR reaction using the commercially available primers and procedures recommended by the producer (Agdia Inc, IN, U.S.A.) and as described in Maroon and Zavriev (2002). Amplified products were observed in agarose gels stained with Ethidium bromide under UV light (Sambrook et al., 2001).

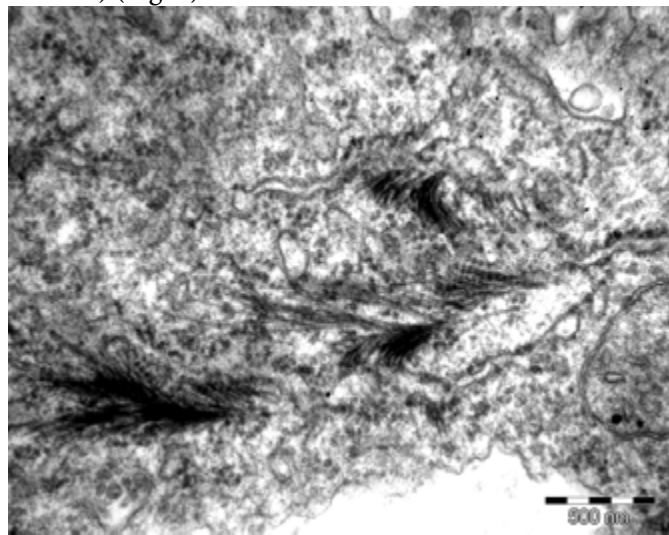
## RESULTS

### Transmission assays:

Mechanical inoculation, grafting, and whiteflies were able to transmit the virus successfully. Soybean seeds from virus infected plants were shriveled and smaller compared to seeds from virus symptom free plants (Fig. 1). The symptoms that developed by grafting (Fig 2A), WFs vectoring (Fig 2B) and mechanical inoculation (Fig. 2C) were similar to those observed in the field. No seed transmission was observed when 200 seeds collected from pods of infected were seeded.

### Electron Microscopy:

Ultrathin sections of diseased tissues showed feather-like inclusions of presumed virions in the cytoplasm, which have been reported before in association with whitefly transmitted *Carlavirus* infections (Brunt et al. 1983) (Fig. 4).



**Figure 4:** Occurrence of feather-like inclusions in the cytoplasm of leaf cells. These types of inclusions were previously reported to be associated with *Carlavirus* infections.

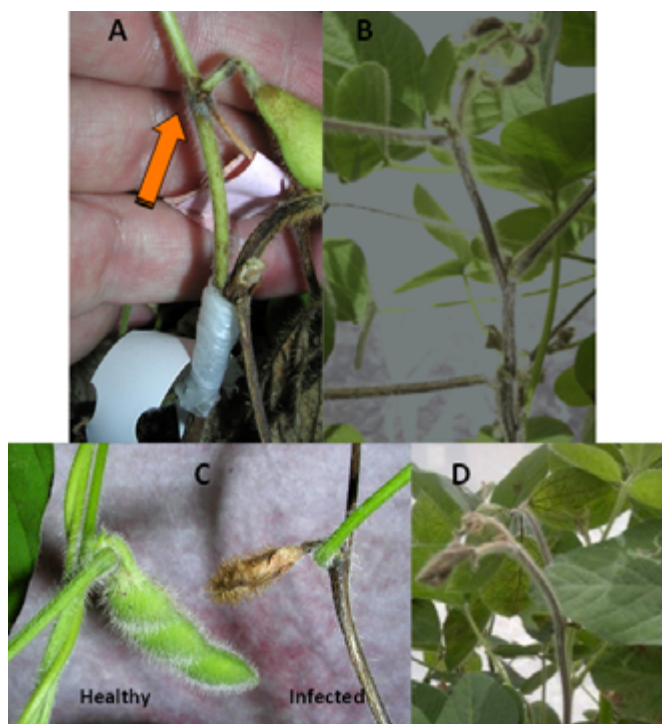
### ELISA test:

Positive ELISA reactions were obtained using the antibody for CPMMV in 8 plants out of the 18 plant species surveyed (Table 1). The ELISA result showed that 36% of whitefly host species surveyed were positive for the virus including cowpea (*Vigna unguiculata* L.), Primrose willow [*Ludwigia octovalvis* (Jacq.) Raven], Horse purslane (*Trianthema portulacastrum* L.) Erect

**Figure 1:** Field symptoms. Above. Symptomatic pods (left) and stem dieback. Below. Seeds from healthy (right bottom) and symptomatic plants (right top).



**Figure 2:** Transmission of the virus by grafting (A), whitefly transmitted (B) and mechanical inoculation (C and D). Pods from healthy (C-left) and symptomatic plants (C-right). Development of symptoms started 21 days after the mechanical inoculation and was obvious a month after inoculation.



spiderling (*Boerhavia erecta* L.), *Ipomea* sp., Yellow thistle (*Argemone mexicana* L.), and Black nightshade (*Solanum americanum* Mill.). However, except the soybeans, none of these host plants showed visual symptoms of the *Carlavirus* except being infested with WFs. The level of *Carlavirus* infection varied from 25-50% (Table 1).

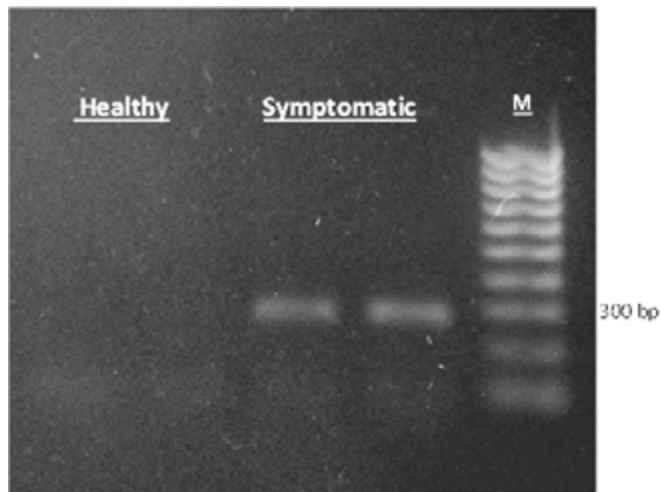
### RT-PCR:

Using specific primers available for *Carlavirus*, a fragment of the expected size (~300bp) was amplified only from plants that gave positive results in the ELISA test and symptomatic soybean plants collected either from the field or from the experimental transmission assays (Fig. 3). In addition, the use of universal primers



for potyvirus (Langeveld et al., 1991) and ELISA tests for other viruses (like SMV, *Potyvirus*) showed negative results.

**Figure 3:** Agarose gel showing the product of RT-PCR using carlavirus specific primers (Agdia). Healthy tissues (two left lines) and symptomatic tissues (two right lines) from plants experimentally transmitted. M =Molecular marker (HiperLadder IV, Bioline)



**Table 1:** Host plants screened with ELISA for Carlavirus detection at Dow AgroSciences Research Station, Santa Isabel, Puerto Rico, during spring 2010.

Scientific name	Family	ELISA result	# plants tested	% Incidence
<i>Ludwigia octovalvis</i> (Jacq.)	Onagraceae	+	4	25
<i>Solanum americanum</i> Mill.	Solanaceae	+	4	25
<i>Trianthema portulacastrum</i> L.	Aizoaceae	+	4	25
<i>Ipomea</i> sp.	Convolvulaceae	+	4	25
<i>Boerhavia erecta</i> L.	Nyctaginaceae	+	4	25
<i>Argemone Mexicana</i> L.	Papaveraceae	+	4	50
<i>Euphorbia heterophylla</i> L.	Euphorbiaceae	–	4	0
<i>Amaranthus dubius</i> Mart.	Amaranthaceae	–	4	0
<i>Malvaceae</i> sp.	Malvaceae	–	4	0
<i>Datura stramonium</i> L.	Solanaceae	–	4	0
<i>Cleome gynandra</i> L.	Cleomaceae	–	4	0
<i>Kallstromia maxima</i> L.	Zygophyllaceae	–	4	0
<i>Macroptilium lathyroides</i> (L.) Urban	Fabaceae	–		0
<i>Vigna unguiculata</i> L.	Fabaceae	+	8	25
<i>Lycopersicon esculentum</i> L.	Solanaceae	–	4	0
<i>Cucurbita</i> sp.	Cucurbitaceae	–	4	0
<i>Ipomoea batatas</i> (L.) Lam.	Convolvulaceae	–	4	0
<i>Glycine max</i> (L.) Merr.	Fabaceae	+	8	25

## DISCUSSION

The ELISA results showed that the antibody used to detect the *Cowpea mild mottle virus* (CpMMV) successfully detected a *Carlavirus* both from the positive controls and host plants surveyed. This indicates that this *Carlavirus* virus is an isolate of the CpMMV, which

supports earlier reports of Almeida et al. (2005) and Tavassoli et al. (2009). The CpMMV was first reported by Brunt and Kenten (1973) on cowpea in Ghana and latter the problem was reported from several tropical regions of Africa and Asia in diverse range of plant species including legumes and solanaceous crops (Rodriguez and Arneodo, 2004). At DAS Research Station farms in Puerto Rico, cowpea is widely grown as a cover crop to protect the soil from erosion and enhance soil fertility. The CpMMV symptoms were observed in soybean before the cowpea was used as a cover crop in the area. The plant species that showed positive results in the ELISA test are grown both within the soybean fields (internal sources of inoculums) and outside the crop fields (external sources). Similarly Harris (1983) mentioned that the source of the virus for spreading into crops could be found among plants in the crop, which could be either weeds or cultivated plants (internal sources) or outside borders, i.e. virus infected wild and cultivated plants (external sources). Although both the cowpea and other host plants that showed positive reaction in the ELISA test did not show any visual symptoms of virus infection, they are used as alternate hosts both for the vectors and the virus. This suggests that the virus may not show the same symptom in different host plants. Our findings are supported by similar findings of Tavassoli et al. (2009), who reported that the CpMMV did not show any visible symptoms in some host plants like *Vigna radiata*, *V. aconitifolia*, and tomato, although they were infected and gave positive results in the ELISA test for the virus. In our hands, we could not find cases of natural infection of tomatoes by CpMMV. These differences in the expression of symptoms in infected plants will make assessment of the disease severity and incidence and its management very difficult. The prevalence of the disease in Puerto Rico is very high. During May-June 2010, the second author has observed a 100% infection in many soybeans lines planted by Illinois Crop Improvement Association at Juana Diaz, Puerto Rico. In the present study we have collected leaf samples only from those host plants that were infested with WFs, but still there may be more plant species that might harbor the virus. Hence, it may be necessary to survey more cultivated and uncultivated species that are commonly grown in the area and screen for the presence of the CpMMV. Integrated approaches should be used to control the disease, which may include control of the vectors using appropriate insecticides, controlling weeds and other alternate hosts or shifting soybean planting sites to areas where WFs and those weeds are less problematic. In general, the results from transmission assays, electron microscopy and ELISA tests indicate that the disease affecting the soybean nurseries in Puerto Rico is caused by an isolate of CpMMV.

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